amendments are of a minor nature and find support in the original application. No new matter is added.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from Deposit Account No. 01-2508/11362.0015.DVUS01.

The Examiner is invited to contact the undersigned attorney at (713) 787-1438 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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Date: May 9, 2002

MARKED UP VERSION OF AMENDMENTS

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Figure 23 shows the coding sequence of the D9D10 L10 diabody

bp 1- 351 : V_H D9D10

bp 352 - 381 : $(G_4S)_2$ linker

bp 382 - 702 : V_L D9D10 (SEQ ID NO 92)

Figure 24 shows the amino acid sequence of the D9D10 L5 diabody

aa 1- 117 : V_H D9D10

aa 118 - 122 : G_4S linker

aa 123 - 229 : V_L D9D10

aa 230 - 235 : His6-tag 5SEQ ID NO 93)

Figure 25 shows the coding sequence of the D9D10 L5 diabody

bp 1- 351 : V_H D9D10

bp 352 - 366 : G_4S linker

bp 367 - 687 : V_L D9D10 (SEQ ID NO 94)

Figure 26 shows the interaction of humanized L5 D9D10 diabody (= crude lysate from *E. Coli*) with IFN γ using SPR analysis. The assay is performed as described in example 5.

Figure 27 shows the coding sequence of the D9D10 L0 triabody

bp 1- 351 : V_H D9D10

bp 352 - 672 : V_L D9D10 (SEQ ID NO 101)

Figure 28 shows the amino acid sequence of the D9D10 L0 triabody

aa 1- 117 : V_H D9D10

aa 118 - 224 : V_L D9D10

aa 225 - 230 : His6-tag (SEQ ID NO 102)

Figure 29 shows the interaction of humanized L0 D9D10 triabody (= crude lysate from *E. Coli*) with IFNγ using SPR analysis. The assay is performed as described in example 6.

Figure 30 shows the neutralization of IFN-gamma-induced MHC class II upregulation on human primary keratinocytes by D9D10 or D9D10 scFv. Human keratinocytes were cultured for 24 h with or without (not shown) 100 U/ml huIFN-gamma in the absence or the presence of D9D10 (2μg/ml). Resting human keratinocytes do not express MHC class II. IFN-gamma induces expression of MHC class II in the keratinocytes and

to generate diabodies, as for example described by Holliger et al. (1993), Poljak (1994) and Zhu et al. (1996), can be used. The generation of diabodies comprising the variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application.

It should also be clear that the scFv's, chimeric antibodies and diabodies described above are not limited to comprise the variable domain of the monoclonal antibody D9D10 but may also comprise variable domains of other anti-IFNγ antibodies, such as the sheep anti-IFNγ antibody described further in the present application, which efficiently neutralize the bioactivity of IFNγ.

Furthermore, the diabodies described above may also comprise two scFv's of different specificities. For example, the latter diabodies may simultaneously neutralize IFNγ on the one hand and may target another molecule, such as TNF-α, IL-1, IL-2, B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, tumour growth factor-beta (TGF-β), transferrin receptor, insulin receptor and prostaglandin E2 or any other molecule, on the other hand.

The present invention also concerns multivalent antibodies which bind and neutralize IFNγ. As used herein, the term multivalent antibody refers to any IFNγ-binding and IFNγ-neutralizing molecule which has more than two IFNγ-binding regions. Examples of such multivalent antibodies are triabodies, tetravalent antibodies, peptabodies and hexabodies which bind and neutralize IFNγ and which have three, four, five and six IFNγ-binding regions, respectively.

The present invention thus relates, as indicated above, to triabodies which bind and neutralize IFNγ. As used herein, the term "triabody" relates to trivalent constructs comprising 3 scFv's, and thus comprising 3 variable domains, as described by Kortt et al. (1997) and Iliades et al. (1997). A method to generate triabodies is described by Kortt et al. (1997) and the generation of triabodies comprising the variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application. It should be noted that the triabodies of the present invention may comprise: 3 variable domains of 3 different anti-IFNγ Ab's (i.e. 3 anti-IFNγ Ab's which recognize and bind a different epitope on IFNγ [see also above]), 3 variable domains of 3 identical

human IFNγ with the carboxylic groups of the dextran layer, the sensorchip was pretreated with 4 cycles of EDC/NHS - thus reducing the amount of unblocked carboxylic groups remaining on the sensor surface - before immobilizing D9D10. Then, immobilization of D9D10 was carried out using a continuous flow of 5µl/min on a sensor chip surface initially activated with 17µl of an 0.05M NHS/ 0.2M EDC mixture. 35µl of typically 3µg/ml D9D10 was injected over the activated surface.Residual unreacted ester groups were blocked by injecting 17µl of 0.1M ethanolamine pH 8.5. D9D10 was immobilised directly on a CM5 chip at an optimal concentration of 3µg/ml in an acetate buffer pH 5.4 resulting in an immobilization level of about 600 RU. Most accurate affinity data were obtained by injecting human IFNγ and monitoring the subsequent binding of scFvD9D10; the latter interacting with remaining free epitopes on human IFNγ. On and off rates were calculated using the BIAevaluation software (Biacore AB).

Results of a typical experiment are shown in figure 3 for murine scFvD9D10 and in figure 4 for humanized scFvD9D10 (These data were generated in separate experiments). Calculated data were in good agreement. As off rates were hardly detectable for both constructs in most experiments, only on rates are shown for the concentrations tested. These data clearly indicated that the humanization did not hamper the binding characteristics of the scFv fragment.

Monoclonal antibodies were generated against the humanized scFvD9D10. A female BALB/c mouse was immunized (injected ~~intraperitoneallly~~ intraperitoneally) 3 times with humanized scFvD9D10 (*i.e., at days 0 (50 µg), 32(25 µg) and 56(25 µg)*). Three months after, a final boost of 25 µg was given. Three days after this last injection, spleen cells were retrieved from the immunized mouse and used for cell fusion. Dissociated splenocytes from the immunized mouse were fused with murine myeloma cells SP2/0-Ag14 (ATCC, CRL-1581) at a ratio of 10:3 using a polyethylene glycol/DMSO solution mainly according the procedure as described by Köhler and Milstein (1975). The fused cells were mixed up and resuspended in DMEM medium supplemented with hypoxanthine, sodium pyruvate, glutamine, a non-essential amino acid solution, 20% heat-inactivated fetalclone (Hyclone Lab., Utah) and 10% BM-Condimed (Boehringer Mannheim). The cells were then distributed to 96 well plates to which aminopterin was

Antisense strand oligos :

IG8178 5'-CTCTGGGTCAGCTCGATGTCCGAGAGTATGACTGAGGCAC-3'
(SEQ ID NO 47)

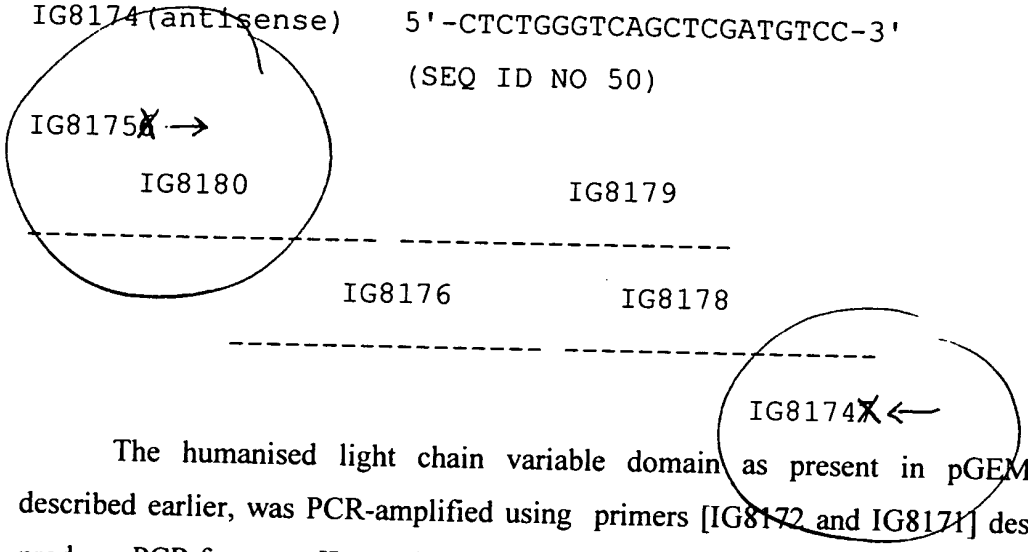
IG8176 5'-TGATTAGCAGGAAGCTGAAAATCTGCACTTGAAAATCCAT-3'
(SEQ ID NO 48)

PCR amplification primers :

XbaI

IG8175(sense) 5'-GTCCCCCGGGTACCTCTAGAATG-3'
(SEQ ID NO 49)

IG8174(antisense) 5'-CTCTGGGTCAGCTCGATGTCC-3'
(SEQ ID NO 50)



The humanised light chain variable domain as present in pGEM-T-V_LH, described earlier, was PCR-amplified using primers [IG8172 and IG8171] designed to produce PCR fragment II containing the complete variable domain cDNA with exception of the last 3 amino acids (IKR), and flanked at the 3'-terminus by an XhoI-cloning site.

IG8172(sense) 5'-GACATCGAGCTGACCCAGAGCCCGGCG-3'
(SEQ ID NO 51)

XhoI

IG8171(antisense) 5'-CGCGCTCGAGTTTGGTACCCTG-3'
(SEQ ID NO 52)

Fusion of the two DNA fragments PCR-I (Ldr) and PCR-II (V_{Lh}) , having 20 bp overlap, was performed by overlap PCR using primerset IG8175 and IG8171. The resulting PCR-III fragment was directly cloned in pGEM-T resulting in the pGEMLdrV_{Lh} plasmid.

Sense strand oligos :

XbaI

IG8180 5'-GTCCCCCGGGTACCTCTAGAATGGATTTTCAAGTGCAGAT-3'

(SEQ ID NO 45)

IG8179 5'-TTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATACTCTCG-3'

(SEQ ID NO 46)

Antisense strand oligos :

IG8177 5'-CTCTGCACCAGCTGCACCTGCGAGAGTATGACTGAGGCAC-3'

(SEQ ID NO 55)

IG8176 5'-TGATTAGCAGGAAGCTGAAAATCTGCACTTGAAAATCCAT-3'

(SEQ ID NO 48)

PCR amplification primers :

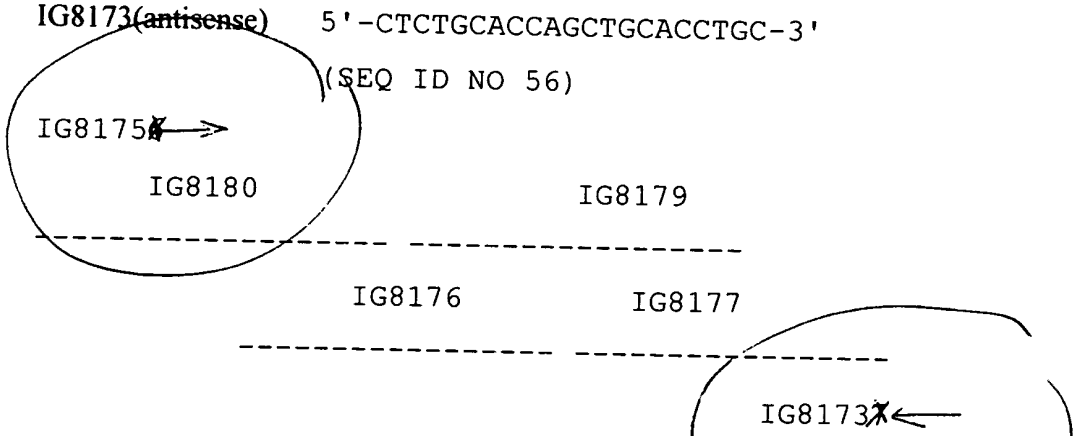
XbaI

IG8175(sense) 5'-GTCCCCCGGGTACCTCTAGAATG-3'

(SEQ ID NO 49)

IG8173(antisense) 5'-CTCTGCACCAGCTGCACCTGC-3'

(SEQ ID NO 56)



The humanised variable heavy chain domain as present in pGEM-T-V_HH, described earlier, was PCR-amplified using primers (IG8168 and IG8167) designed to produce PCR-VI fragment containing the complete variable domain cDNA, and flanked at the 3'-terminus by an XhoI-cloning site.

IG8168(sense) 5'-CAGGTGCAGCTGGTGCAGAGCGGTAG-3'

(SEQ ID NO 57)

expression plasmids, named pEE12hD9D10 and pEE14hD9D10 then consists of the pEE-backbone plasmid containing the GS-selection unit, carrying the light chain fusion gene expression casette followed by a comparable heavy chain fusion gene expression casette.

The approach of assembling a single expression plasmid containing separate transcription units for both heavy and light chains and the selectable marker is ~~adviced~~ in order to ensure coamplification with the marker gene.

A schematic representation of both plasmids is given in figures 5 and 6.

The cDNA sequence encoding the complete humanized D9D10 heavy chain fusion protein is given in figure 7. (SEQ ID NO 66)

The cDNA sequence encoding the humanized D9D10 light chain fusion protein is given in figure 8. (SEQ ID NO 68)

The amino acid sequence of the humanized D9D10 heavy chain fusion protein is given in figure 9. (SEQ ID NO 67)

The aminoacid sequence of the humanized D9D10 light chain fusion protein is given in figure 10. (SEQ ID NO 69)

* *Small scale expression of humanized ~~D9D10~~ chimeric antibody in COS cells*

A quick way to determine the feasibility of expressing a recombinant protein in mammalian cells and to evaluate its ~~functionallity~~ functionality is transient expression of the product in COS cells (Gluzmann, 1981). COS cells are Simian Virus 40 (SV40)-permissive CV1 cells (African monkey kidney) stably transformed with an origin-defective SV40 genome, thereby constitutively producing the SV40 T-antigen. In SV40-permissive cells, T-antigen initiates high copy number transient episomal replication of any DNA-vector that contains the SV40 origin of DNA replication. Both the pEE12 and pEE14 expression vectors contain an SV40 origin of replication in the SV40 early promoter region controlling the GS-selection gene, and thus permits efficient transient expression in COS cells.

Small amounts of functionally active antibody were made by transient expression in COS cells. COS7 cells (ATCC CRL 1651) were routinely cultured in DMEM supplemented with ~~0,03%~~ 0.05% glutamine and 10% fetal calf serum. For preparative scale transfection, an optimized DEAE-transfection protocol (McCutchan, 1968) was used.

Alternatively, other well known transfection methods such as Ca-phosphate precipitation, electroporation, liposome-based transfection can be used. Briefly, exponentially growing COS7 cells were seeded in cell factories (Nunc, Rochester, NY, USA) at 3.5 10^4 cells/cm^2 about 18 h before transfection, after which the cells were washed twice with MEM-Hepes pH 7.1 (Gibco, Rockville, MD, USA) and allowed to cool to bench temperature. ~~0,5~~ 0.5 µg/cm^2 cell surface of high quality plasmid DNA (CsCl-density purification) of the mammalian expression plasmids pEE12hD9D10 and pEE14hD9D10 was ethanol precipitated, redissolved in 25 µl/cm^2 MEM-Hepes pH 7.1 and slowly added to the same volume of 2 mg/ml DEAE-dextran MW 500.000 (Pharmacia) in MEM-Hepes pH 7.1. The DNA-DEAE-dextran precipitate (50 µl/cm^2) was allowed to form for 20-25 min, put on the cells for 25 min and removed to be stored at -20°C (the same precipitate can be reused in a second transfection experiment with ~~the~~ same efficiency).

The cells were incubated during the next 3.5 hours in DMEM growth medium (Gibco) containing 0.1 mM chloroquine (Sigma) (~~0,3~~ 0.3ml /cm^2) in a CO_2-incubator ~~at~~ 37°C, then washed two times with growth medium and further incubated for 18 hrs in complete culture medium enriched with 0.1 mM sodium butyrate (Sigma) at 37°C (~~0,3~~ 0.3ml /cm^2). The next day the cells were washed twice with serum free DMEM medium supplemented with 0.03% glutamine (Merck) and then incubated for 48h (~~determined in analytical scale~~ experiments as the optimal harvest time) in 150 µl/cm^2 cell surface of the same medium at 37°C, after which conditioned medium was harvested and stored at -70°C ~~until~~ until purification. As negative control COS cells were also transfected with the empty expression vectors pEE12 and pEE14.

Quality control of the crude CM was performed by IFNγ-binding assay in ELISA format, by SPR-analysis and by measuring the inhibition of IFNγ mediated MHC class II-induction.

Human Interferonγ-coating Elisa

96 well ELISA culture plates (Nunc 469914) were coated with 100 ng/well hIFNγ (Genzyme 80-3348-01, 1mg/ml) diluted in 50mM TrisHCl pH8.5, 150 mM NaCl, by 18h incubation at 4°C. Blocking of nonspecific binding was performed in PBS/0.1% caseine (200µl/well, 1h ,37°C). All washing steps were performed with PBS/0.05% Tween-20 (3

role in septic shock. We generated data in a mouse model system using an anti-mouse IFNγ called F3 (Froyen et al., 1995).

The generalized Shwartzman model is a lethal shock syndrome in experimental animals which is elicited by 2 consecutive injections of LPS. In the laboratory of prof. Billiau (Rega Institute, Catholic University Leuven, Belgium), such a model was developed in mice (Billiau et al., 1987). At time 0, the mice were injected with 5 μg LPS into the footpad, followed 24 h later by a second intravenous injection of 100 μg. Morbidity and mortality was scored for 5 days. Untreated animals normally died within 2 days after the second injection. Mice pretreated with the anti-muIFNγ antibody F3 were completely protected against the lethal effect and only showed moderate disease symptoms. This protection could be achieved with as little as 2.4 μg F3 given 24h before the first injection. In order to score the severity of the disease, the symptoms were classified in 5 groups :

Score 0 : not sick or mild piloerection

Score 1 : piloerection and diarrhoea

Score 2 : hemorhagic conjunctivitis and bleeding at the mouth and anus

Score 3 : paralysis of the hind legs

Score 4 : death

The highest score that could be obtained is 4. Since the number of mice in each group was relatively low (5), we established a limit of the disease score (=2) that had to be reached in the saline group in order to be a representative experiment.

The schedule we used in order to compare F3 and its scFv in this Shwartzman model was as follows: NMRI mice were given the preparative dose of 5 μg LPS at time 0. At the time points +6h, +12h and +23h the mice were injected ip with 190 μg scFvF3 (Froyen et al., 1995) or 30 μg F3. Control animals were given saline at the same time points. Each group consisted of 5 mice. The mice were given a score according to the above mentioned classification.

In the first experiment, 40% more mice were protected in the scFvF3 group when compared with the control group. A second experiment was set up using a slightly adapted protocol: an additional injection was given at timepoint +3h. The result of this experiment (shown in table) was similar to that of experiment 1 in that 40% more mice

independent experiments are shown in figure 33. The mice treated with scFvF3 were better protected against the cachectic effect than the control mice.

These results also indicate that scFvF3 antibody fragments do have a protective effect of cachexia but to a lesser extent than the parental F3 antibody. Although results were promising, it was clear that the effect of the scFv fragment was limited either due to its fast clearance or to lowered affinity. Optimization of the injection schedule was needed to obtain comparable results.

8.5. Beneficial effects in septic shock in non-human primates

The best documented sepsis model in non-human primates is the one in which baboons are given lethal infusions of *E.coli*. As described by Creasey et al. (1991), response to lethal *E.coli* challenge occurs in 3 stages: an inflammatory stage marked by a fall in white blood cell count (0-2 hr) and the appearance in plasma of TNFα, IL-1β and IL-6; a coagulant stage marked by a fall in fibrinogen concentration (2-6 hr); and a hypoxic cell injury stage marked by a rise in SGPT/BUN and by a gradual cardiovascular collapse, and death (6-24 hr).

Since the baboon animal model was not readily available, we are establishing a comparable rhesus monkey model. D9D10 and derived constructs interacted well with rhesus IFNγ as determined in an antiviral bioassay (set up as described in example ~~7.2.~~ 8.2.).

Septic shock can be induced by infusion either of life bacteria or of endotoxin in sedated monkeys. After administration of different concentrations of the ~~D9D10~~ anti-hIFNγ derivatives, several parameters are monitored including :

- mortality (should be 100% in control (non-treated) group)
- pathophysiology
- serum concentration of cytokines such as TNFα, IL-1 and IL-6 using ELISA or bioassay (Villinger et al., 1993)
- endotoxin profile using the limulus amoebocyte lysate assay

8.6. Beneficial effects during experimental autoimmune encephalomyelitis in non-human primates

A. Pharmacokinetics of D9D10 and derivatives in monkey and effect on hIFNγ clearance

The clearance of the antibody derivatives is of importance as molecules with a slow clearance have a prolonged efficacy. This implicates that less material has to be injected which is better for the patient and which is cost effective, especially when a longer treatment period is advisable. Therefore, complexes of IFNγ and D9D10 derivatives are used in clearance studies in non-human primates as a prerequisite to guide further *in vivo* studies in these animals.

The clearance of D9D10, scFvD9D10H6, D9D10 MOTAB I and D9D10 MOTAB II , is monitored after a bolus injection in healthy marmoset. Specific ELISA's are used for monitoring; no labelling of the antibody constructs is required.

Blood clearance of radiolabelled marmoset IFNγ after a bolus intravenous injection alone or in combination with one of the antibody constructs are also performed.

B. Beneficial effects of the D9D10 antibody constructs on EAE in non-human primates

In order to evaluate the therapeutic potential of the anti-IFNγ Mab D9D10 and derivatives, we are testing this antibody in a relevant non-human primate model for MS as the final step in our preclinical research. This model is required since the antibody is not cross-reacting with IFNγ from rodents and the biological activity of IFNγ is very species specific (huIFNγ is not active on cells other than human or non-human primates (Terrell and Green, 1993)). D9D10 and derived constructs interact well with marmoset IFNγ as determined in an antiviral bioassay (set up as described in example ~~7.2~~ 8.2.) and using surface plasmon resonance (set up as described in example 1).

The EAE model is chosen as it is a generally accepted model for Multiple Sclerosis. We opt for the EAE model in common marmoset (*Callithrix jacchus*) as it is well developed (Massacesi et al., 1995; Genain et al., 1995), it has a pathology of MR-detectable lesions which reflects those in MS and the model shows a high incidence of EAE induction with a chronic progressive/relapsing-remitting course.

Acute PK-Tox

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Figure 28 shows the amino acid sequence of the D9D10 L0 triabody

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aa 225 - 230 : His6-tag (SEQ ID NO 102)

Figure 29 shows the interaction of humanized L0 D9D10 triabody (= crude lysate from *E. Coli*) with IFNγ using SPR analysis. The assay is performed as described in example 6.

Figure 30 shows the neutralization of IFN-gamma-induced MHC class II upregulation on human primary keratinocytes by D9D10 or D9D10 scFv. Human keratinocytes were cultured for 24 h with or without (not shown) 100 U/ml huIFN-gamma in the absence or the presence of D9D10 (2μg/ml). Resting human keratinocytes do not express MHC class II. IFN-gamma induces expression of MHC class II in the keratinocytes and

to generate diabodies, as for example described by Holliger et al. (1993), Poljak (1994) and Zhu et al. (1996), can be used. The generation of diabodies comprising the variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application.

It should also be clear that the scFv's, chimeric antibodies and diabodies described above are not limited to comprise the variable domain of the monoclonal antibody D9D10 but may also comprise variable domains of other anti-IFNγ antibodies, such as the sheep anti-IFNγ antibody described further in the present application, which efficiently neutralize the bioactivity of IFNγ.

Furthermore, the diabodies described above may also comprise two scFv's of different specificities. For example, the latter diabodies may simultaneously neutralize IFNγ on the one hand and may target another molecule, such as TNF-α, IL-1, IL-2, B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, tumour growth factor-beta (TGF-β), transferrin receptor, insulin receptor and prostaglandin E2 or any other molecule, on the other hand.

B2

The present invention also concerns multivalent antibodies which bind and neutralize IFNγ. As used herein, the term multivalent antibody refers to any IFNγ-binding and IFNγ-neutralizing molecule which has more than two IFNγ-binding regions. Examples of such multivalent antibodies are triabodies, tetravalent antibodies, peptabodies and hexabodies which bind and neutralize IFNγ and which have three, four, five and six IFNγ-binding regions, respectively.

The present invention thus relates, as indicated above, to triabodies which bind and neutralize IFNγ. As used herein, the term "triabody" relates to trivalent constructs comprising 3 scFv's, and thus comprising 3 variable domains, as described by Kortt et al. (1997) and Iliades et al. (1997). A method to generate triabodies is described by Kortt et al. (1997) and the generation of triabodies comprising the variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application. It should be noted that the triabodies of the present invention may comprise: 3 variable domains of 3 different anti-IFNγ Ab's (i.e. 3 anti-IFNγ Ab's which recognize and bind a different epitope on IFNγ [see also above]), 3 variable domains of 3 identical

human IFNγ with the carboxylic groups of the dextran layer, the sensorchip was pretreated with 4 cycles of EDC/NHS - thus reducing the amount of unblocked carboxylic groups remaining on the sensor surface - before immobilizing D9D10. Then, immobilization of D9D10 was carried out using a continuous flow of 5µl/min on a sensor chip surface initially activated with 17 µl of an 0.05M NHS/ 0.2M EDC mixture. 35µl of typically 3µg/ml D9D10 was injected over the activated surface. Residual unreacted ester groups were blocked by injecting 17 µl of 0.1M ethanolamine pH 8.5. D9D10 was immobilised directly on a CM5 chip at an optimal concentration of 3 µg/ml in an acetate buffer pH 5.4 resulting in an immobilization level of about 600 RU. Most accurate affinity data were obtained by injecting human IFNγ and monitoring the subsequent binding of scFvD9D10; the latter interacting with remaining free epitopes on human IFNγ. On and off rates were calculated using the BIAevaluation software (Biacore AB).

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B3

Monoclonal antibodies were generated against the humanized scFvD9D10. A female BALB/c mouse was immunized (injected intraperitoneally) 3 times with humanized scFvD9D10 (*i.e., at days 0 (50 µg), 32(25 µg) and 56(25 µg)*). Three months after, a final boost of 25 µg was given. Three days after this last injection, spleen cells were retrieved from the immunized mouse and used for cell fusion. Dissociated splenocytes from the immunized mouse were fused with murine myeloma cells SP2/0-Ag14 (ATCC, CRL-1581) at a ratio of 10:3 using a polyethylene glycol/DMSO solution mainly according the procedure as described by Köhler and Milstein (1975). The fused cells were mixed up and resuspended in DMEM medium supplemented with hypoxanthine, sodium pyruvate, glutamine, a non-essential amino acid solution, 20% heat-inactivated fetalclone (Hyclone Lab., Utah) and 10% BM-Condimed (Boehringer Mannheim). The cells were then distributed to 96 well plates to which aminopterin was added 24 hours after the cell fusion. Each well contained between 1 to 5 growing

Antisense strand oligos :

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(SEQ ID NO 47)

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(SEQ ID NO 48)

PCR amplification primers :

XbaI

IG8175(sense) 5'-GTCCCCCGGGTACCTCTAGAATG-3'
(SEQ ID NO 49)

IG8174(antisense) 5'-CTCTGGGTCAGCTCGATGTCC-3'
(SEQ ID NO 50)

IG8175→

IG8180 IG8179

IG8176 IG8178

IG8174←

B4

The humanised light chain variable domain as present in pGEM-T-V_LH, described earlier, was PCR-amplified using primers [IG8172 and IG8171] designed to produce PCR fragment II containing the complete variable domain cDNA with exception of the last 3 amino acids (IKR), and flanked at the 3'-terminus by an XhoI-cloning site.

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Fusion of the two DNA fragments PCR-I (Ldr) and PCR-II (V_{Lh}) , having 20 bp overlap, was performed by overlap PCR using primerset IG8175 and IG8171. The resulting PCR-III fragment was directly cloned in pGEM-T resulting in the pGEMLdrV_{Lh} plasmid.

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(SEQ ID NO 55)

IG8176 5'-TGATTAGCAGGAAGCTGAAAATCTGCACTTGAAAATCCAT-3'

(SEQ ID NO 48)

PCR amplification primers :

XbaI

IG8175(sense) 5'-GTCCCCCGGGTACCTCTAGAATG-3'

(SEQ ID NO 49)

IG8173(antisense) 5'-CTCTGCACCAGCTGCACCTGC-3'

(SEQ ID NO 56)

IG8175→

IG8180 IG8179

---------- ----------

IG8176 IG8177

---------- ----------

IG8173←

The humanised variable heavy chain domain as present in pGEM-T-V_HH, described earlier, was PCR-amplified using primers (IG8168 and IG8167) designed to produce PCR-VI fragment containing the complete variable domain cDNA, and flanked at the 3'-terminus by an XhoI-cloning site.

IG8168(sense) 5'-CAGGTGCAGCTGGTGCAGAGCGGTAG-3'

(SEQ ID NO 57)

expression plasmids, named pEE12hD9D10 and pEE14hD9D10 then consists of the pEE-backbone plasmid containing the GS-selection unit, carrying the light chain fusion gene expression casette followed by a comparable heavy chain fusion gene expression casette.

The approach of assembling a single expression plasmid containing separate transcription units for both heavy and light chains and the selectable marker is advised in order to ensure coamplification with the marker gene.

A schematic representation of both plasmids is given in figures 5 and 6.

The cDNA sequence encoding the complete humanized D9D10 heavy chain fusion protein is given in figure 7. (SEQ ID NO 66)

The cDNA sequence encoding the humanized D9D10 light chain fusion protein is given in figure 8. (SEQ ID NO 68)

The amino acid sequence of the humanized D9D10 heavy chain fusion protein is given in figure 9. (SEQ ID NO 67)

The aminoacid sequence of the humanized D9D10 light chain fusion protein is given in figure 10. (SEQ ID NO 69)

B6

* *Small scale expression of humanized D9D10 chimeric antibody in COS cells*

A quick way to determine the feasibility of expressing a recombinant protein in mammalian cells and to evaluate its functionallity is transient expression of the product in COS cells (Gluzmann, 1981). COS cells are Simian Virus 40 (SV40)-permissive CV1 cells (African monkey kidney) stably transformed with an origin-defective SV40 genome, thereby constitutively producing the SV40 T-antigen. In SV40-permissive cells, T-antigen initiates high copy number transient episomal replication of any DNA-vector that contains the SV40 origin of DNA replication. Both the pEE12 and pEE14 expression vectors contain an SV40 origin of replication in the SV40 early promoter region controlling the GS-selection gene, and thus permits efficient transient expression in COS cells.

Small amounts of functionally active antibody were made by transient expression in COS cells. COS7 cells (ATCC CRL 1651) were routinely cultured in DMEM supplemented with 0.03% glutamine and 10% fetal calf serum. For preparative scale transfection, an optimized DEAE-transfection protocol (McCutchan, 1968) was used.

Alternatively, other well known transfection methods such as Ca-phosphate precipitation, electroporation, liposome-based transfection can be used. Briefly, exponentially growing COS7 cells were seeded in cell factories (Nunc, Rochester, NY, USA) at 3.5 10^4 cells/cm^2 about 18 h before transfection, after which the cells were washed twice with MEM-Hepes pH 7.1 (Gibco, Rockville, MD, USA) and allowed to cool to bench temperature. 0.5 µg/cm^2 cell surface of high quality plasmid DNA (CsCl-density purification) of the mammalian expression plasmids pEE12hD9D10 and pEE14hD9D10 was ethanol precipitated, redissolved in 25 µl/cm^2 MEM-Hepes pH 7.1 and slowly added to the same volume of 2 mg/ml DEAE-dextran MW 500.000 (Pharmacia) in MEM-Hepes pH 7.1. The DNA-DEAE-dextran precipitate (50 µl/cm^2) was allowed to form for 20-25 min, put on the cells for 25 min and removed to be stored at -20°C (the same precipitate can be reused in a second transfection experiment with the same efficiency).

The cells were incubated during the next 3.5 hours in DMEM growth medium (Gibco) containing 0.1 mM chloroquine (Sigma) (0.3ml /cm^2) in a CO_2-incubator at 37°C, then washed two times with growth medium and further incubated for 18 hrs in complete culture medium enriched with 0.1 mM sodium butyrate (Sigma) at 37°C (0.3ml /cm^2). The next day the cells were washed twice with serum free DMEM medium supplemented with 0.03% glutamine (Merck) and then incubated for 48h (determined in analytical scale experiments as the optimal harvest time) in 150 µl/cm^2 cell surface of the same medium at 37°C, after which conditioned medium was harvested and stored at -70°C until purification. As negative control COS cells were also transfected with the empty expression vectors pEE12 and pEE14.

Quality control of the crude CM was performed by IFNγ-binding assay in ELISA format, by SPR-analysis and by measuring the inhibition of IFNγ mediated MHC class II-induction.

Human Interferonγ-coating Elisa

96 well ELISA culture plates (Nunc 469914) were coated with 100 ng/well hIFNγ (Genzyme 80-3348-01, 1mg/ml) diluted in 50mM TrisHCl pH8.5, 150 mM NaCl, by 18h incubation at 4°C. Blocking of nonspecific binding was performed in PBS/0.1% caseine (200µl/well, 1h ,37°C). All washing steps were performed with PBS/0.05% Tween-20 (3

role in septic shock. We generated data in a mouse model system using an anti-mouse IFNγ called F3 (Froyen et al., 1995).

The generalized Shwartzman model is a lethal shock syndrome in experimental animals which is elicited by 2 consecutive injections of LPS. In the laboratory of prof. Billiau (Rega Institute, Catholic University Leuven, Belgium), such a model was developed in mice (Billiau et al., 1987). At time 0, the mice were injected with 5 μg LPS into the footpad, followed 24 h later by a second intravenous injection of 100 μg. Morbidity and mortality was scored for 5 days. Untreated animals normally died within 2 days after the second injection. Mice pretreated with the anti-muIFNγ antibody F3 were completely protected against the lethal effect and only showed moderate disease symptoms. This protection could be achieved with as little as 2.4 μg F3 given 24h before the first injection. In order to score the severity of the disease, the symptoms were classified in 5 groups :

Score 0 : not sick or mild piloerection

Score 1 : piloerection and diarrhoea

Score 2 : hemorhagic conjunctivitis and bleeding at the mouth and anus

Score 3 : paralysis of the hind legs

Score 4 : death

The highest score that could be obtained is 4. Since the number of mice in each group was relatively low (5), we established a limit of the disease score (=2) that had to be reached in the saline group in order to be a representative experiment.

The schedule we used in order to compare F3 and its scFv in this Shwartzman model was as follows: NMRI mice were given the preparative dose of 5 μg LPS at time 0. At the time points +6h, +12h and +23h the mice were injected ip with 190 μg scFvF3 (Froyen et al., 1995) or 30 μg F3. Control animals were given saline at the same time points. Each group consisted of 5 mice. The mice were given a score according to the above mentioned classification.

In the first experiment, 40% more mice were protected in the scFvF3 group when compared with the control group. A second experiment was set up using a slightly adapted protocol: an additional injection was given at timepoint +3h. The result of this experiment (shown in table) was similar to that of experiment 1 in that 40% more mice

independent experiments are shown in figure 33. The mice treated with scFvF3 were better protected against the cachectic effect than the control mice.

These results also indicate that scFvF3 antibody fragments do have a protective effect of cachexia but to a lesser extent than the parental F3 antibody. Although results were promising, it was clear that the effect of the scFv fragment was limited either due to its fast clearance or to lowered affinity. Optimization of the injection schedule was needed to obtain comparable results.

8.5. Beneficial effects in septic shock in non-human primates

The best documented sepsis model in non-human primates is the one in which baboons are given lethal infusions of *E.coli*. As described by Creasey et al. (1991), response to lethal *E.coli* challenge occurs in 3 stages: an inflammatory stage marked by a fall in white blood cell count (0-2 hr) and the appearance in plasma of TNFα, IL-1β and IL-6; a coagulant stage marked by a fall in fibrinogen concentration (2-6 hr); and a hypoxic cell injury stage marked by a rise in SGPT/BUN and by a gradual cardiovascular collapse, and death (6-24 hr).

Since the baboon animal model was not readily available, we are establishing a comparable rhesus monkey model. D9D10 and derived constructs interacted well with rhesus IFNγ as determined in an antiviral bioassay (set up as described in example 8.2).

Septic shock can be induced by infusion either of life bacteria or of endotoxin in sedated monkeys. After administration of different concentrations of the D9D10 anti-hIFNγ derivatives, several parameters are monitored including :

- mortality (should be 100% in control (non-treated) group)
- pathophysiology
- serum concentration of cytokines such as TNFα, IL-1 and IL-6 using ELISA or bioassay (Villinger et al., 1993)
- endotoxin profile using the limulus amoebocyte lysate assay

8.6. Beneficial effects during experimental autoimmune encephalomyelitis in non-human primates

A. Pharmacokinetics of D9D10 and derivatives in monkey and effect on hIFNγ clearance

The clearance of the antibody derivatives is of importance as molecules with a slow clearance have a prolonged efficacy. This implicates that less material has to be injected which is better for the patient and which is cost effective, especially when a longer treatment period is advisable. Therefore, complexes of IFNγ and D9D10 derivatives are used in clearance studies in non-human primates as a prerequisite to guide further *in vivo* studies in these animals.

The clearance of D9D10, scFvD9D10H6⁻, D9D10 MOTAB I and D9D10 MOTAB II , is monitored after a bolus injection in healthy marmoset. Specific ELISA's are used for monitoring; no labelling of the antibody constructs is required.

Blood clearance of radiolabelled marmoset IFNγ after a bolus intravenous injection alone or in combination with one of the antibody constructs are also performed.

B. Beneficial effects of the D9D10 antibody constructs on EAE in non-human primates

In order to evaluate the therapeutic potential of the anti-IFNγ Mab D9D10 and derivatives, we are testing this antibody in a relevant non-human primate model for MS as the final step in our preclinical research. This model is required since the antibody is not cross-reacting with IFNγ from rodents and the biological activity of IFNγ is very species specific (huIFNγ is not active on cells other than human or non-human primates (Terrell and Green, 1993)). D9D10 and derived constructs interact well with marmoset IFNγ as determined in an antiviral bioassay (set up as described in example 8.2) and using surface plasmon resonance (set up as described in example 1).

The EAE model is chosen as it is a generally accepted model for Multiple Sclerosis. We opt for the EAE model in common marmoset (*Callithrix jacchus*) as it is well developed (Massacesi et al., 1995; Genain et al., 1995), it has a pathology of MR-detectable lesions which reflects those in MS and the model shows a high incidence of EAE induction with a chronic progressive/relapsing-remitting course.

Acute PK-Tox